UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

DEC 21 1999

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Registration of Reynoutria sachalinensis Bioprotectant (EPA File Symbol 072179-R) Containing 100% Dried Ground Giant Knotweed (Reynoutria sachalinensis) as its Active Ingredient and an Non-Food End-use Product Milsana Bioprotectant Concentrate (EPA File Symbol 072179-E) containing 5% Reynoutria sachalinensis Extract, to Control Powdery Mildews in Green Houses. Chemical No. 055809; Case No. 065908 Review of Product Chemistry Data. MRID Nos. 448219-01, -02, -03, and 448573-01; Submission No.: S565442; DP Barcode: D257772

FROM: Freshteh Toghrol, Ph.D., Senior Scientist F. Toghrol
Biochemical Pesticides Branch
Biopesticides & Pollution Prevention Division

TO: Driss Benmhend, Regulatory Action Leader
Biochemical Pesticides Branch
Biopesticides & Pollution Prevention Division

ACTION R REQUESTED

KHH BioSci, Inc., requests registration of a technical grade active ingredient Reynoutria sachalinensis Bioprotectant (EPA File Symbol 072179-R) Containing 100% of dried and ground Giant Knotweed (Reynoutria sachalinensis) and an end-use product Milsalna Bioprotectant Concentrate (EPA File Symbol 072179-E) containing 5% ethanolic extract of dried and ground Reynoutria sachalinensis and 95% of inert ingredients. The end-use product is a non-food formulation to be used in green houses to control powdery mildews.

To support this registration, KHH BioSci has submitted 2 proposed label and 2 Confidential Statements of Formula dated 4/9/99 for each basic formulation (EPA Symbol No. 072179-R) and (EPA File Symbol 072179-E) . The registrant has also submitted product chemistry data (MRID Nos.448219-01, -02, -03, and 448573-01).

Inert ingredient information may be entitled to confidential treatment

BPPD CONCLUSIONS AND RECOMMENDATIONS

1. The submitted product chemistry data do not satisfy the data requirements for product identity and composition (GLN 151-10) for Reynoutria sachalinensis Bioprotectant (EPA File Symbol 072179-R) containing 100% of dried and ground Giant knotweed plant material and the end-use product Milsalna Bioprotectant Concentrate (EPA File Symbol 072179-E) containing 5% ethanolic extract of dried and ground Reynoutria sachalinensis and 95% of inert ingredients. The registrant must clarify, what is the technical grade active ingredient (TGAI). According to CSFs and physical chemical characteristics, there are two TGAIs. The first TGAI is a dried and ground Reynoutria sachalinensis and the second TGAI is ethanolic extract of dried and ground Reynoutria sachalinensis. However, the registrant did not submit any product chemistry data (151-10 to 151-17) for the second TGAI (the ethanolic extract of Reynoutria sachalinensis).

2a. The submitted product chemistry data not satisfy the data requirements for manufacturing process (GLN 151-11) of TGAI containing 100% dried ground plant material of giant Knotweed. There is no quality control monitoring of the dry plant material and no discussion of monitoring for plant or soil pathogen in the dry ground plant material.

2b. The submitted product chemistry data do not satisfy the data requirements for manufacturing process (GLN 151-11) of the end-use product containing 5% of *Reynoutria sachalinensis* extract. Description of beginning materials and manufacturing process with adequate discussions of production equipment, volumes, and methods. Also, it is not clear, how the nominal concentration (5%w/w) of the ethanolic extract in each formulation was measured.

3. The submitted data do not satisfy the data requirement for discussion of formation of impurities (151-12). The registrant must adequately discuss the potential for toxic soil or plant pathogens in raw plant material, as well as effect of storage on producing these pathogen. Additionally, the registrant must adequately discuss the formation of impurities in ethanolic plant extract, and the end-use product.

4. The preliminary analysis (151-13) data requirement is not satisfy. The 5 batch analysis of 5 different batches of Reynoultria sachalinensis ethanolic extract is required to show consistency of production of the TGAI extract. Additionally, 5 batch analysis of 5% w/w TGAI in the end-use product is required. The registrant must explain clearly how 5% of R. sachalinensis extract w/w (TGAI) is measured or calculated in end-use product, for quality control and quality assurance.

5. The certification of ingredient limits (151-15) data requirement is not satisfied. The CSF for TGAI and the end-use product must be prepared from 5 batch analysis of TGAI extract and the end-use product. If TGAI is the ethanolic extract, then product chemistry data (151-10 to 151-17), a revised CSF and revised label for new TGAI is required.

6. The analytical method (151-16) submitted is a biological assay, an indirect effect of Milsana for control of cucumber powdery mildew is acceptable. The registrant has indicated GC or HPLC will be used for [redacted], which is acceptable However, the registrant must submit the analytical method, that measures the total R. sachalinensis ethanolic extract (TGAI). This is the method used to quantified 5 batch analysis (151-13) in the

TGAI extract, and then, the method that measure the 5% of this extract that is present in the end-use product .

7. The physical chemical characteristics for end-use product is acceptable. However, the registrant must submit data or rational for solubily, vapor pressure, dissociation constant, octanol/water partition coefficient, and pH of TGAI (dried, ground plant material). Also physical chemical characteristics are required for the ethanolic plant extract, which is the real technical grade active ingredient (ethanolic extract), which was used to formulate the end-use product.

8. The product is a non-food use on all ornemental species in green houses to control powdery mildews. mold (Botrytis cinerea). Therefore, exemption from the requirement of a tolerance or numeric establishment of a tolerance in or on all food commodities is not considered

9. The mode of action of giant knotweed extract is to boost the ornemental plants natural defense mechanisms in the plant against certain fungal diseases (by increasing up to 5 times the phenolic material in the plant).

10. The product is a non-food use on all ornemental species in green house to control powdery mildews, therefore, ecological toxicity studies were not submitted.

(151-17) Physical and Chemical Property Assessment

The submitted physical and chemical characteristics for Reynoultria sachalinensis Bioprotectant (EPA File Symbol 072179-R) containing 100% dried ground giant knotweed (Reynoultria sachalinensis) as technical grade active ingredient and end-use product Milsalna Bioprotectant Concentrate (EPA File Symbol 072179-E) containing 5% Reynoultria sachalinensis Extract are summarized below:

Guideline No.151B-17	**TGAI (MRID No. 448219-02)**	**End-use product (MRID No. 448**
color	Olive green, brown and cream	N/R
Physical state	Non-uniform solid, various size of leaves and twigs	Liquid
Odor	No odor	N/R
Melting point	N/A	N/R
Density, specific gravity	0.135 g/mL	1.394 g/mL
Solubility	Not submitted	N/R
Vapor pressure	Not submitted	N/R
Dissociation constant	Not submitted	N/R
Octanol/water partition coefficient	Not submitted	N/R
pH	Not submitted	6.84 ± 0.03 for 1% solution
Stability	N/R	N/R
Oxidizing/reducing action	N/R	Not oxidizing/reducing, except for a
Flammability	N/R	No flashpoint up to 101.8 C
Explodability	*N/R	Do not contain explosive ingredient
Storage Stability	N/R	The study is in progress
Viscosity	*N/R	79.4 cp at 20.1 C
Miscibility	*N/R	Not emulsifiable
Corrosion characteristics	N/R	Do not contains corrosive ingredien

*N/A = not applicable because, the product by nature does not have any of above characteristics.
N/R= data are not required for the product

Not submitted = data are required for the TGAI, but were not submitted.

The physical chemical characteristics for end-use product is acceptable. However, the registrant must submit data or rational for solubily, vapor pressure, dissociation constant, octanol/water partition coefficient, and pH of TGAI (dried, grounded R.S.). The registrant must also submit physical chemical characteristics for ethanolic plant extract, which is the real technical grade active ingredient used to formulate the end-use product.

cc: Freshteh Toghrol, Driss Benmhend, BPPD Subject file.
F. Toghrol, CS#1: BPPD: Tel (703) 308-7014: 12/21/99

[redacted]

DATA EVALUATION REPORT

MILSANA® (*REYNOUTRIA SACHALINENSIS BIOPROTECTANT*)

STUDY TYPES: Product Identity and Disclosure of Ingredients (OPPTS 880.1100)
Description of Beginning Materials &
Manufacturing Process (OPPTS 880.1200)
Discussion of Formation of Impurities (OPPTS 880.1400)
Certification of Ingredient Limits (OPPTS 830.1750)
MRID 44821901

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 30-A2

Primary Reviewer:
Robin Brothers, Ph.D., D.A.B.T.

Signature: Robin A. Brothers
Date: NOV 24 1999

Secondary Reviewers:
Sylvia Milanez, Ph.D., D.A.B.T.

Signature:
Date: NOV 24 1999

Robert H. Ross, Group Leader

Signature: Robert H. Ross
Date: NOV 24 1999

Quality Assurance:
Eric Lewis, M.S.

Signature: Eric B. Lewis
Date: NOV 24 1999

Disclaimer

This Data Evaluation Report may have been altered by the Biopesticides and Pollution Prevention Division subsequent to signing by Oak Ridge National Laboratory personnel.

Oak Ridge National Laboratory, managed by Lockheed Martin Energy Research Corp. for the U.S. Department of Energy under contract number DE-AC05-96OR22464.

Milsana
MRID No. 44821901

Product Identity and Disclosure of Ingredients (OPPTS 880.1100)
Description of Beginning Materials & Manufacturing Process (OPPTS 880.1200)
Discussion of Formation of Impurities (OPPTS 880.1400)
Preliminary Analysis (OPPTS 830.1700)
Certification of Ingredient Limits (OPPTS 830.1750)

EPA Reviewer: Freshteh Toghrol, Ph.D.
Biopesticides and Pollution Prevention Division (7511W)

F. Toghrol, Date 12/21/99

DATA EVALUATION RECORD

STUDY TYPES:

Product Identity and Disclosure of Ingredients (OPPTS 880.1100)
Description of Beginning Materials &
Manufacturing Process (OPPTS 880.1200)
Discussion of Formation of Impurities (OPPTS 880.1400)
Certification of Ingredient Limits (OPPTS 830.1750)

CASE NO: 065908

PC CODE: 055809

DP BARCODE: D257772

SUBMISSION: S565442

MRID NO: 44821901

TEST MATERIAL:

1. Reynoultria sachalinensis Bioprotectant (EPA File Symbol No. (072179-R) a TGAI containing 100% dried ground plant material(Giant knotweed).

2. Milsana® Bioprotectant Concentrate (EPA File Symbol No. 072179-E) an end-use product containing 5% of *Reynoutria sachalinensis* extract in denatured alcohol plus 95% of other ingredients.

SYNONYMS: *Reynoutria sachalinensis* extract, Giant knotweed, *Polygonum sachalinense*

STUDY NUMBER: KHH-99-002

SPONSOR: KHH BioSci, Inc., P.O. Box 13169, Research Triangle Park, NC 27709

TESTING FACILITY: None

Manufacturing process information may be entitled to confidential treatment

TITLE OF REPORT: Manufacturing Data Requirements Supporting Milsana® bioprotectant Concentrate and *Reynoutria sachalinensis* bioprotectant

AUTHOR: Karen Ferrand

REPORT ISSUED: April 9, 1999

EXECUTIVE SUMMARY:

The product identity, ingredients, certified ingredient limits, manufacturing process, preliminary analysis, and discussion of formation of impurities for Milsana® bioprotectant are given in MRID 44821901.

1. The technical grade active ingredient is Reynoultria sachalinensis Bioprotectant (EPA File Symbol No. (072179-R) consists entirely of the dried and ground plant material from harvested Reynoultria sachalinensis plants grown for this purpose. The production of Milsana® (*Reynoutria sachalinensis* bioprotectant) begins with the growing and harvesting the *Reynoutria sachalinensis* or giant knotweed plants. After the material is dry it is milled to a coarse flake, which is 100% TGAI.

[redacted]

Classification of the study -

Product Identity and Disclosure of Ingredients: **Unacceptable** , but upgradeable with clarification of the technical grade active ingredient (also see attachment I BioSci letter to Mr. Benmhend dated 9/22/98, containing refferences to public litrature).

Description of Beginning Materials and Manufacturing Process: **Unacceptable** but upgradeable with adequate discussions of production equipment, volumes, and methods.

Discussion of Formation of Impurities: **Unacceptable** but upgradeable with discussion of the potential for toxic soil or plant pathogens in the raw plant material.

Preliminary Analysis: **Unacceptable** but upgradeable with preliminary analysis of the Technical Grade Active Ingredient

Certification of Ingredient Limits: **Unacceptable** but upgradeable with clarification of the technical grade active ingredientin the ethanolic axtract.

COMPLIANCE: Signed and dated Data Confidentiality Statements and GLP statements were provided. GLP Statements stated that the study did not meet the requirements of 40 CFR Part 160 because it is not currently required to do so. No Quality Assurance Statements were provided.

Inert ingredient information may be entitled to confidential treatment

Manufacturing process information may be entitled to confidential treatment

Milsana
MRID No. 44821901

Product Identity and Disclosure of Ingredients (OPPTS 880.1100)
Description of Beginning Materials & Manufacturing Process (OPPTS 880.1200)
Discussion of Formation of Impurities (OPPTS 880.1400)
Preliminary Analysis (OPPTS 830.1700)
Certification of Ingredient Limits (OPPTS 830.1750)

A. PRODUCT IDENTITY AND DISCLOSURE OF INGREDIENTS (OPPTS 880.1100)

[redacted]

B. MANUFACTURING PROCESS (OPPTS 880.1200)

[redacted]

Manufacturing process information may be entitled to confidential treatment

Inert ingredient information may be entitled to confidential treatment

[redacted]

C. DISCUSSION OF FORMATION OF IMPURITIES (OPPTS 880.1400)

No reactions are utilized in the processing, only physical blending, extraction, and distillation. The product is produced and stored under conditions to minimize degradation (conditions are not specifically described). The product is a complex mixture of natural components. There is no discussion of monitoring for plant or soil pathogens in the dry ground plant material. Improper field handling or drying could result in growth of pathogens that may be of toxicological significance, such as Aspergillus.

D. PRELIMINARY ANALYSIS OF SAMPLES (OPPTS 830.1700)

No preliminary analysis was provided. The registrant states that the product is not manufactured by an integrated system and therefore this is not required. The registrant does not clarify if the active ingredient in Milsana®, *Reynoutria sachalinensis*, is a registered product or will be registered as a TGAI. There were no materials provided to the reviewer stating what the active chemical component in the ethanol extract of *Reynoutria sachalinensis* may be.

E. CERTIFICATION OF INGREDIENT LIMITS (OPPTS 830.1750)

[redacted]

F. DISCUSSION

The product identity, ingredients, certified ingredient limits, manufacturing process, preliminary analysis, and discussion of formation of impurities for Milsana® are given in MRID 44821901. The active ingredient of Milsana® is an extract of the giant knotweed plant, *Reynoutria sachalinensis*. Milsana® uses an aqueous liquid fertilizer as the vehicle/solvent. The technical material provided with for Basfoliar® 36 Extra lists the application recommendations for the fertilizer by crop, fruit and vegetable type. These variations in use of the vehicle may need to be considered when determining final use concentrations of Milsana® and there may be a limit of useability due to possible phytotoxicity of the vehicle. The author states that the manufacturing-use product and the TGAI are the same; that being the dried and ground *Reynoutria sachalinensis* plant material itself (pg 5/34). This statement is confusing as the plant material is extracted and semi-purified before use in the end-use product. In MRID 44857301, the efficacy test of Milsana for control of downy mildew, a "tea" extract was also used. The TGAI should be more clearly defined as to its chemical nature. The product identity states that the *Reynoutria sachalinensis* extract is the active ingredient. It is important to note that there are no discussions of the toxicological significance of Milsana® or of the plant material itself

G. STUDY DEFICIENCIES

The liquid fertilizer is described by the example product Basfoliar® 36 Extra but there are no statements that this will be the only liquid fertilizer used or that the formulation of Basfoliar® 36 Extra meets a certain set of specifications that any other liquid fertilizer would have to meet. There was no Confidential Statement of Formula supplied with this material. The equipment used to produce the product was not adequately described. Average batch or production size was not described. There are no statements as to how often the quality tests are performed on the product. The text on formation of impurities states that the temperature is controlled to prevent degradation of the product but those controls and temperature limits are not mentioned specifically. There is no discussion on quality control monitoring of the dry plant material and no discussion of monitoring for plant or soil pathogens in the dry ground plant material. No preliminary analysis was provided but the registrant states that the product is not manufactured by an integrated system and therefore this is not required. The registrant does not clarify if the active ingredient in Milsana®, *Reynoutria sachalinensis*, is a registered product or will be registered as a TGAI. There were no materials provided to the reviewer stating what the active

chemical component in the ethanol extract of *Reynoutria sachalinensis* may be. The introduction states that *Reynoutria sachalinensis* is the TGAI but the product identity states the *Reynoutria sachalinensis* extract is the active ingredient of Milsana®.

Classification of the Study:

Product Identity and Disclosure of Ingredients: **Unacceptable** but upgradeable with clarification of the technical grade active ingredient.
Description of Beginning Materials and Manufacturing Process: **Unacceptable** but upgradeable with adequate discussions of production equipment, volumes, and methods.
Discussion of Formation of Impurities: **Unacceptable** but upgradeable with discussion of the potential for soil or plant pathogens in the raw plant material.
Preliminary Analysis: **Unacceptable** but upgradeable with preliminary analysis of the technical grade active ingredient (TGAI) extract.
Certification of Ingredient Limits: **Unacceptable** but upgradeable with clarification of the liquid fertilizer ingredient and clarification of the active ingredientin ethanol extract.

DATA EVALUATION REPORT

REYNOUTRIA SACHALINENSIS

STUDY TYPES: PHYSICAL AND CHEMICAL PROPERTIES
MRID 44821902

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 30A2

Primary Reviewer: Robin Brothers, Ph.D., D.A.B.T.	Signature: Date:	Robin A. Brothers NOV 24 1999
Secondary Reviewers: Sylvia Milanez, Ph.D., D.A.B.T.	Signature: Date:	 NOV 24 1999
Robert H. Ross, Group Leader	Signature: Date:	Robert H. Ross NOV 24 1999
Quality Assurance: Eric Lewis, M.S.	Signature: Date:	Eric B. Lewis NOV 24 1999

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Oak Ridge National Laboratory, managed by Lockheed Martin Energy Research Corp. for the U.S. Department of Energy under contract number DE-AC05-96OR22464.

EPA Reviewer: Freshteh Togrohl
Biopesticides and Pollution Prevention Division (7511W)

F. Togrohl Date 12/21/99

DATA EVALUATION REPORT

STUDY TYPES:

Color (OPPTS 830.6302)
Physical State (OPPTS 830.6303)
Odor (OPPTS 830.6304)
Bulk Density (OPPTS 830.7300)

CASE NO: 065908

PC CODE: 055809

DP BARCODE: D257772

SUBMISSION: S565442

MRID NO: 44821902

TEST MATERIAL: *Reynoutria sachalinensis*

SYNONYMS: Giant knotweed, *Polygonum sachalinense*

STUDY NUMBER: 5483-F(01)

SPONSOR: KHH BioSci, Inc., P.O. Box 13169, Research Triangle Park, NC 27709

TESTING FACILITY: Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110-2299

TITLE OF REPORT: Selected Group B Analyses for Dried Plant Material of *Reynoutria sachalinensis*, Lot # 11-89/2A

AUTHOR: John Cookinham

REPORT ISSUED: March 16, 1999

EXECUTIVE SUMMARY: The color, physical state, odor, and density for *Reynoutria sachalinensis*, giant knotweed plant material, are given in MRID 44821902. Only these selected chemical and physical properties were presented. MRID 44821901

states that the plant material is a Technical Grade Active Ingredient but no other assessments pertinent to the TGAI were presented or discussed.

Classification of the study -
Color: **Acceptable**
Physical State: **Acceptable**
Odor: **Acceptable**
Bulk Density: **Acceptable**
Other assessments that may be pertinent to a TGAI or MUP were not provided.

COMPLIANCE: Signed and dated Data Confidentiality Statements and GLP statements were provided. A Quality Assurance Statement was provided.

A. PHYSICAL AND CHEMICAL PROPERTIES

All tests were made on Lot number 11-89/2A, dried plant material

Color (OPPTS 830.6302): Olive green, brown and cream, at 20.3 °C by visual inspection.

Physical State (OPPTS 830.6303): Non-uniform solid, various sizes of leaves and twigs, observed at 20.3 °C.

Odor (OPPTS 830.6304): No odor was detected during the course of other testing.

Bulk Density (OPPTS 830. 7300): 0.135 g/mL, tapped weight using a calibrated graduate cylinder (CIPAC MT-3, Specific Gravity and Density).

B. DISCUSSION

The color, physical state, odor, and bulk density for *Reynoutria sachalinensis* plant material are given in MRID 44821902. All tests were adequately documented. MRID 44821901 states that the plant material is a Technical Grade Active Ingredient but additional tests as would be required for a TGAI or MUP were not reported in MRID 44821902. The laboratory study protocol (pg. 12/14) states that characterization, solubility, and stability of the test substance were the responsibility of the sponsor and were not included in this report.

C. STUDY DEFICIENCIES

Other assessments that may be pertinent to a TGAI or MUP were not provided.
Classification of the Study:
Color: **Acceptable**
Physical State: **Acceptable**
Odor: **Acceptable**
Bulk Density: **Acceptable**
Other assessments that may be pertinent to a TGAI or MUP were not provided.

DATA EVALUATION REPORT

MILSANA® BIOPROTECTANT CONCENTRATE (*REYNOUTRIA SACHALINENSIS*)

STUDY TYPES: PHYSICAL AND CHEMICAL PROPERTIES (OPPTS 830.6303-7300)
MRID 44821903

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 30A2

Primary Reviewer: Robin Brothers, Ph.D., D.A.B.T.	Signature: Date:	Robin A. Brothers NOV 24 1999
Secondary Reviewers: Sylvia Milanez, Ph.D., D.A.B.T.	Signature: Date:	 NOV 24 1999
Robert H. Ross, Group Leader	Signature: Date:	Robert H. Ross NOV 24 1999
Quality Assurance: Eric Lewis, M.S.	Signature: Date:	Eric B. Lewis NOV 24 1999

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EPA Reviewer: Freshteh Togrohl, Ph.D
Biopesticides and Pollution Prevention Division (7511W)

F. Togrohl, Date 12/21/99

DATA EVALUATION REPORT

STUDY TYPES:
Physical State (OPPTS 830.6303)
Density (OPPTS 830.7300)
pH (OPPTS 830.7000)
Oxidizing/reducing Action (OPPTS 830.6314)
Flammability (OPPTS 830.6315)
Viscosity (OPPTS 830.7100)

CASE NO: 065908

PC CODE: 055809

DP BARCODE: D257772

SUBMISSION: S565442

MRID NO: 44821903

TEST MATERIAL: Milsana® Bioprotectant Concentrate (*Reynoutria sachalinensis*) BAS 114UBF, Lot # AF 455-79-1

SYNONYMS: Giant knotweed, *Polygonum sachalinense*

STUDY NUMBER: 5483-F(02)

SPONSOR: KHH BioSci, Inc., P.O. Box 13169, Research Triangle Park, NC 27709

TESTING FACILITY: Midwest Research Institute, 425 Volker Boulevard, Kansas City, Missouri 64110-2299

TITLE OF REPORT: Selected Group B Analyses for Milsana® Bioprotectant Concentrate, BAS 114UBF, Lot #AF 455-79-1

AUTHOR: John Cookinham

REPORT ISSUED: March 16, 1999

EXECUTIVE SUMMARY: The physical state, density, pH, oxidizing/reducing action, flammability, and viscosity for Milsana® Bioprotectant Concentrate which contains

Reynoutria sachalinensis, giant knotweed plant material as the active ingredient are given in MRID 44821903. Only these selected chemical and physical properties were presented. Corrosiveness and storage stability were not provided.

Classification of the study -
Physical State (OPPTS 830.6303): **Acceptable**
Density (OPPTS 830.7300): **Acceptable**
pH (OPPTS 830.7000): **Acceptable**
Oxidizing/reducing Action (OPPTS 830.6314): **Acceptable**
Flammability (OPPTS 830.6315): **Acceptable**
Viscosity (OPPTS 830.7100): **Acceptable**
Additional properties that may be required for end-use products such as corrosiveness and storage stability were not provided.

COMPLIANCE: Signed and dated Data Confidentiality Statements and GLP statements were provided. A Quality Assurance Statement was provided.

A. PHYSICAL AND CHEMICAL PROPERTIES

All tests were made on Milsana® Bioprotectant Concentrate BAS 114UBF, Lot # AF 455-79-1

Physical State (OPPTS 830.6303): Liquid by visual inspection at 20.1 °C

Relative Density (OPPTS 830.7300): 1.394 (compared to water) at 25 °C using a pycnometer

pH (OPPTS 830.7000): 6.84 for a 1% w/w solution at 25 °C

Oxidizing/reducing Action (OPPTS 830.6314):
Approximately 5 g of the test substance was weighed and added to each of the five tubes containing the reagent. The tubes were capped and shaken and observed immediately, at 1 hour and after 24 hours of standing at ambient temperatures. No reaction was noted with tap water, hexane, monoammonium phosphate, or potassium permanganate. A mild reaction with zinc consisting of bubbles and heat was noted

Flammability (OPPTS 830.6315): No flashpoint at temperatures up to 101.8 °C by the Penske-Mertens closed cup.

Viscosity (OPPTS 830.7100): 79.4 cP by Brookfield spindle viscometer at 20.1 °C

B. DISCUSSION

The physical state, relative density, pH, flammability, viscosity, and oxidizing reducing action for Milsana® Bioprotectant Concentrate containing *Reynoutria sachalinensis* plant material are given in MRID 44821903. All tests were adequately documented. Additional tests as would be required for an EUP, such as storage stability and corrosivity were not addressed in this report. The laboratory study protocol (pg. 16/21) states that characterization, solubility, and stability of the test substance were the responsibility of the sponsor and were not included in this report.

C. STUDY DEFICIENCIES

Other assessments that may be pertinent to an end-use product were not provided.

Classification of the Study:

Physical State (OPPTS 830.6303): **Acceptable**
Density (OPPTS 830.7300): **Acceptable**
pH(OPPTS 830.7000): **Acceptable**
Oxidizing/reducing Action (OPPTS 830.6314): **Acceptable**
Flammability (OPPTS 830.6315): **Acceptable**
Viscosity (OPPTS 830.7100): **Acceptable**
Additional properties that may be required for end-use products such as corrosiveness and storage stability were not provided.

ATTACHMET I

KHH BioSci, Inc.

Mr. Driss Benmhend
U.S. Environmental Protection Agency
Biopesticides and Pollution Prevention Div.
401 M Street, N.W.
Washington, D.C. 20460

Sept. 22, 1998

Subject: Milsana® bioprotectant - Characterization of TGAI

Dear Mr. Benmhend,

As discussed in our phone conversations of August 25th and September 1st, 1998, I have prepared a summary document for you and the members of your team regarding the investigations into the active ingredient of *Reynoutria sachalinensis* (giant knotweed), the plant which is used to formulate Milsana® bioprotectant. For your reference, we have also included a copy of the preregistration conference material from 4/14/98.

Reynoutria sachalinensis is a native of the buckwheat family and is originally native to east Asia, after which it was introduced to Europe and the United States. The rhizomes, leaves, and stems have been used in the orient as Hu Zhang ("Polygoni rhizoma") and were applied in folk medicine as a laxative, a diuretic, and for the treatment of dermatitis and athlete's foot, etc. In Japan, it is commonly used as a vegetable and has been found to be an excellent source of vitamins A, C ,and E. It was introduced into Europe and subsequently into North America during the 19th century as a fodder plant for cattle. Commercial products prepared from *Rs* extracts have been registered and sold as plant fortifiers in Europe under the name Milsana since 1990.

We consider our technical grade active ingredient (TGAI) to be the dried, ground plant material. As you may recall, the compound or compounds responsible for the activity of *Reynoutria sachalinensis* are not direct fungicides. They appear to be naturally occurring elicitors, which induce the plant's natural immune system to produce phytoalexins, thus providing resistance in the host plant to powdery mildew. The presence of light is necessary for this reaction to occur. There also appears to be a general strengthening effect on a variety of plants characterized by increased chlorophyll content, vigorous growth, and delayed senescence. Efforts by many researchers to isolate the active moieties have failed. The enclosed document summarizes the investigations into the identification of this resistance-inducing factor in extracts of *Reynoutria sachalinensis*, so that you may clearly understand the extent of these efforts. In general, studies have excluded the possibility of polypeptides or proteins, terpenoids, phenolic substances, simple or reducing sugars, flavonoids, or steroids. The consensus now is that the compound is probably somewhat polar, amphoteric, and most likely a carbohydrate with a hydrophobic moiety.

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Since we cannot use conventional chemical analytical methods to perform quality control, a reliable bioassay technique has been developed. Because there is no direct fungitoxic effect on the pathogen and the pathogen is an obligate parasite, living host plant tissues (cucumber plants) must be used. We are in the process of preparing the protocol for an independent laboratory to validate this method for us under GLP conditions. We will submit this for your approval as well.

We have characterized the ground dried plant material as % carbohydrates, fat, ash, protein, and moisture. Further analysis of the plant would most probably not provide any further insight into the identity of the active ingredient, as you will see by reading the attached summary. In view of these particular circumstances, we would propose the preparation of a waiver request for active ingredient identification with supporting data from our bioassay technique as a method to measure biochemical activity.

As you and I have discussed, we are hoping that you can give us some guidance on what efforts you deem necessary in order to describe this material satisfactorily for the registration submission for Milsana bioprotectant.

We would be happy to meet with you, if you feel that it would be helpful to this process.

Best regards,

Karen H. Ferrand

Karen H. Ferrand
KHF/rkn

Enclosures

cc: Mr. Roy Sjoblad
Dr. Russell Jones
Dr. Freshteh Toghrol

September 22, 1998

Milsana® Bioprotectant Concentrate:

Summary of the investigations into the identification of the resistance-inducing factor in extracts of *Reynoutria sachalinensis*

I. Introduction

The active ingredient responsible for the activity of *Reynoutria sachalinensis* is not a direct fungicide. It appears to be a natural elicitor, which induces the plant's natural immune system to produce phytoalexins, thus providing resistance in the host plant. Efforts by many researchers to isolate the active ingredient or ingredients have failed.

The following is a summary of most of these efforts and consists primarily of two papers: E. Ammermann and M. Scherer (1992), and Daayf, Schmitt, and Bélanger (1997) which in turn also contain summaries of other research. All relevant references have been included at the end of this paper.

II. Compounds isolated from *Reynoutria sachalinensis*

Chi, Moon and Lee[3] in a Korean publication from 1983 described the isolation of Physcion, Emodin, its 8-O-ß-D-glucoside and ß-sitosterol from the rhizomes of *Reynoutria sachalinensis*. (Fig. 1)

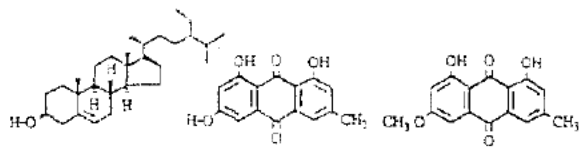


Fig. 1: Structural formulas of Physcion, Emodin, ß-sitosterol

The commercially available chemicals: Emodin, Quecetin, Rutin and ß-sitosterol, were tested for their fungicidal activity against powdery mildew on cucumber, but no significant control was observed, with the exception of Emodin.

However, a thin-layer chromatographic comparison of pure Emodin with the aqueous extract from *Reynoutria sachalinensis* revealed, that no trace of Emodin could be detected in the extract.

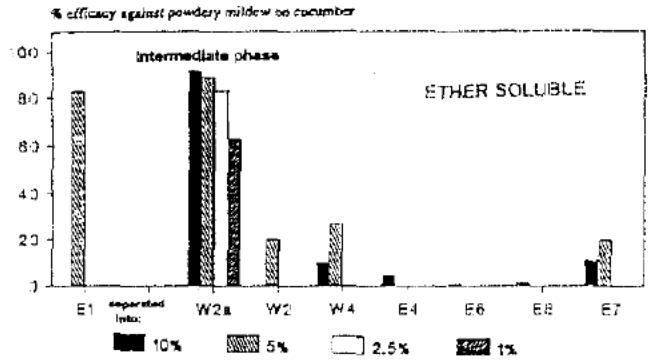
Thus, the first possible candidate, which could have been responsible for the induced fungicidal activity of Milsana, had to be eliminated.

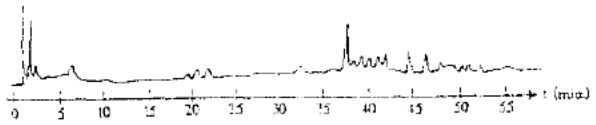
III. *Extraction methods*

a. In the classical extraction[2] procedure, organic solvents with increasing polarity are used, starting from a very nonpolar solvent (either pentane or petrolether in this case) and progressing finally to methanol as a very polar solvent. Checking the biological activity of the fractions very quickly made it clear that the active ingredient must be a very polar component and one which was far from simple to isolate.

b. A more systematic approach started from a concentrated methanol extract of the dried and ground leaves, and followed the ether extraction procedure according to LAATSCH[12]. Following this scheme, it should be possible to make a rough classification of compounds in the different fractions of the extract according to their acid, alkaline and neutral characteristics. (Ph.D. thesis of Dr. Kowalewski)[9]. (Fig. 2 and 3)

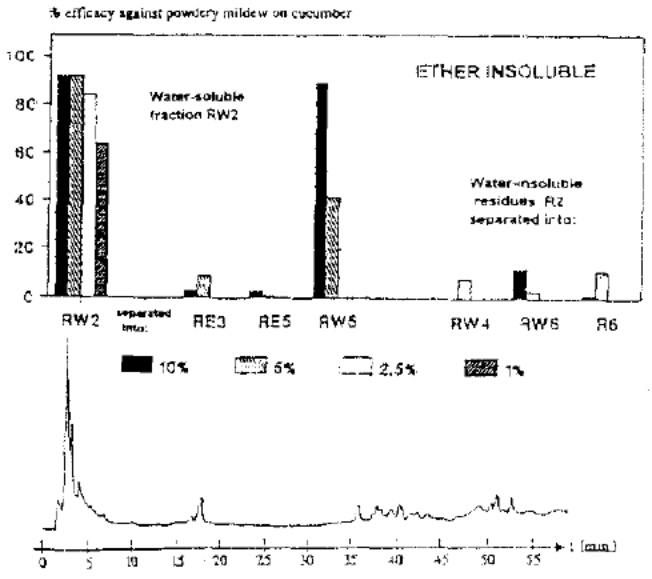
Fig. 2 and 3: acc to the dissertation of Kowalewski





chromatogram of fraction W2a
gradient: 100 % water + 5 % methanol
up to 100 % methanol; 280 nm

Ph.D. thesis Kowalewski, A. K. TH Darmstadt 1992



chromatogram of fraction RW5
gradient: 100 % water + 5 % methanol
up to 100 % methanol; 280 nm

Ph.D. thesis Kowalewski, A. K. TH Darmstadt 1992

Two of the fractions obtained using this procedure retained remarkable resistance-inducing activity when applied to the host plant. One fraction in the ether-soluble part formed a highly stable interphase. Such a phase could be caused by tenside-type compounds, which carry over some of the water-soluble components into the ether phase. The other biologically active fraction came from the ether-insoluble aqueous phase. The chromatographic separation of both phases by HPLC showed profiles looking like forests in the distance but with no distinct peaks, which could be identified in a follow-up program using mass-spectrometric methods.

c. Polarity of the resistance-inducing agent [10]

A bulk transfer (LAATSCH, 1988) was used to separate the extract into its different substance classes. The water extract was extracted with the same volume of ethyl acetate. The different fractions were tested for their efficacy against powdery mildew with the cucumber leaf disc test. Figure 4 shows the efficacy of some fractions from the bulk transfer. The fractions with the highest efficacy (RW5) exclusively contained water-soluble substances like simple sugars and sugar alcohols only.

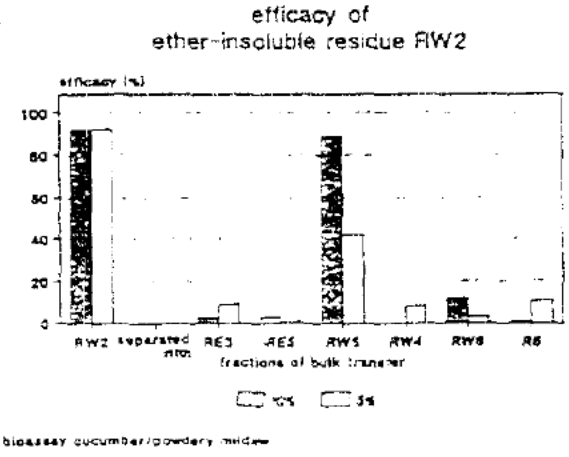


Figure 4: Efficacy of some fractions from the bulk transfer in different concentrations: RW2= ether-insoluble residue, RE3= ether-soluble acids, RE5= ether-soluble bases, RW5= alkali-soluble substances, RW4= water-soluble acids, RW6= alkali-soluble acids, R6 insoluble residue (nomenclature after LAATSCH, 1988).

In contrast to this, an extraction with water and ethyl acetate showed that the activity was distributed equally in the aqueous and the organic phase. So it is unlikely that the resistance-inducing factor is a simple sugar. The contrasting behavior of the solubility indicates a substance with a hydrophilic and a hydrophobic part.

d. Extractions with phenol[2] are used to remove proteins from aqueous extracts. (This included: 1) phenols; 2) $CHCl^3$/phenols 1:1; and 3) twice $CHCl^3$/isoamylalcohols (24/1)). In this case the aqueous extract, treated with phenol, showed the same biological activity as the untreated extract. Thus the active ingredient cannot be attributed to a proteinaceous compound.

e. Another approach to trace the active ingredient was made by Prof. Binger from the Max-Planck-Institut für Kohleforschung (Max Planck Institute for Coal Research) in Mühlheim, Germany. He and his team extracted the dried and ground leaves using liquid supercritical CO^2 as solvent. First crude fractions showed some of the previously mentioned activity against gray mold (*Botrytis cinerea*) but not against powdery mildews. A gas-chromatographic isolation procedure coupled with MS gave clear hints of four steroids. The pure steroids, which are commercially available in reasonable quantities, were rechecked for their biological activity. Not surprisingly in this case, no significant induced fungicidal activity at all was found.

IV. Chromatographic methods

a. Starting from a concentrated methanol extract from the dried and ground leaves, a more detailed study used high-pressure liquid chromatography with a reversed-phase column. Water with a gradient of increasing amounts of methanol was used as solvent. This produced a reasonable separation. Tests of biological activity against powdery mildew or cucumber were somewhat surprising. (Fig. 5a and 5b - Kowalewski, 1992)

Figure 5a

HPLC-chromatogram on DELTAPAK C18
gradient: 100 % water + 5 % methanol
up to 100 % methanol

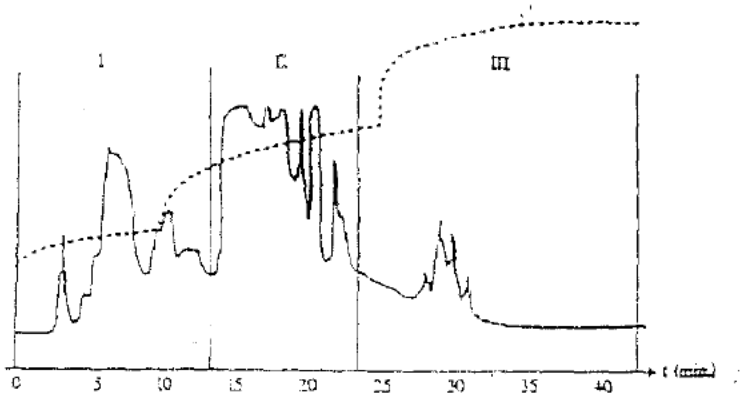
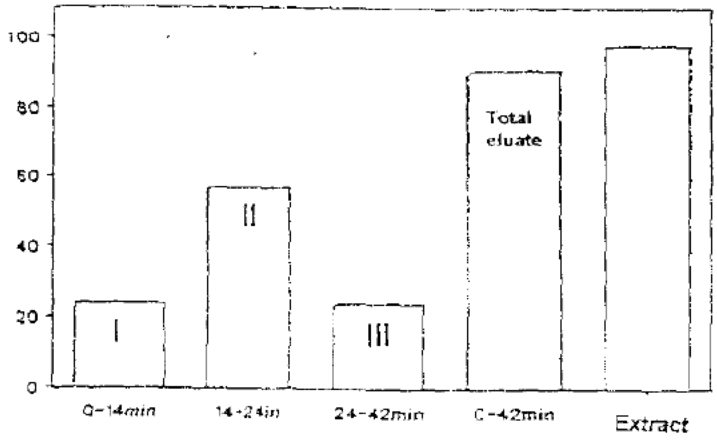


Figure 5b

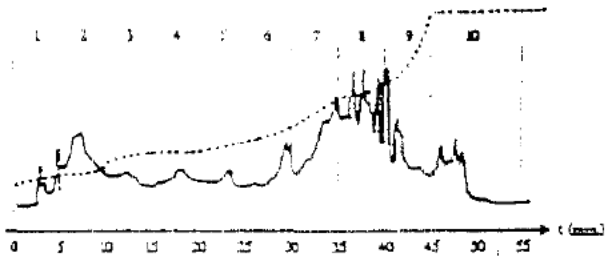
% efficacy against powdery mildew on cucumber



The combined fractions showed the same biological activity as the original extract. But none of the three separated fractions reached this level of activity. Different gradient programs resulted in more or less comparable complex chromatograms. The recheck of the biological activity only revealed a relatively broad dissipation of the active ingredient over many poorly defined fractions.

b HPLC system[10)]

An HPLC system from Millipore and a preparative reversed-phase column (Waters column, DELTAPAK C18, 5 µm, 100 A) was used (detection: 280 nm, flow rate: 3 ml/min). The extract was fractionated with a gradient from 100% water to 100% methanol over a period of 55 minutes. The ten collected fractions (each 15 ml) were tested for their efficacy against powdery mildew with the cucumber leaf disc test. Examination of the extract by HPLC showed that the active ingredient was distributed over the column. Figure 6 shows that the high efficacy of the extract and the combined fractions was not obtained by one single fraction. The activity probably spread over the column. This spreading was also observed on silica thin-layer plates in previous investigations (KOWALEWSKI, 1989).



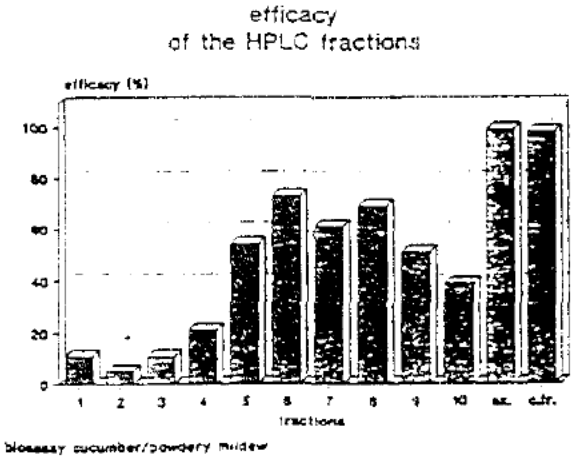


Figure 6: upper part: Chromatogram of *R. sachalinensis* extract fractionated with a RP-column (detection: 280 nm, dotted line: gradient from 100% water to 100% methanol
lower part: Efficacy of the extract (ex.), the combined fractions (c.fr.) and ten single fraction obtained from the HPLC.

V. Chemical modification

Since all these attempts to extract and isolate the active ingredient by chromatography had failed, the following chemical experiments were undertaken to characterize the unknown compound or principle.

a. The aqueous extract from leaves of *Reynoutria sachalinensis* was treated with proteinase K[2] which non-specifically cleaves all kinds of proteins. The extract incubated with proteinase K showed the same fungicidal activity as the untreated one. This result, in combination with the previously mentioned negative result from the phenol extraction, excludes a proteinaceous character for the active ingredient, responsible for the activity of aqueous *Reynoutria* extracts.

b. Hydrolytic treatment[2] of the aqueous extract by boiling it with either hydrochloric acid or sodium hydroxide showed, after neutralization, that the acid treatment increased the biological activity in comparison with the untreated. The alkaline treatment reduced the fungicidal activity. (Figure 7)

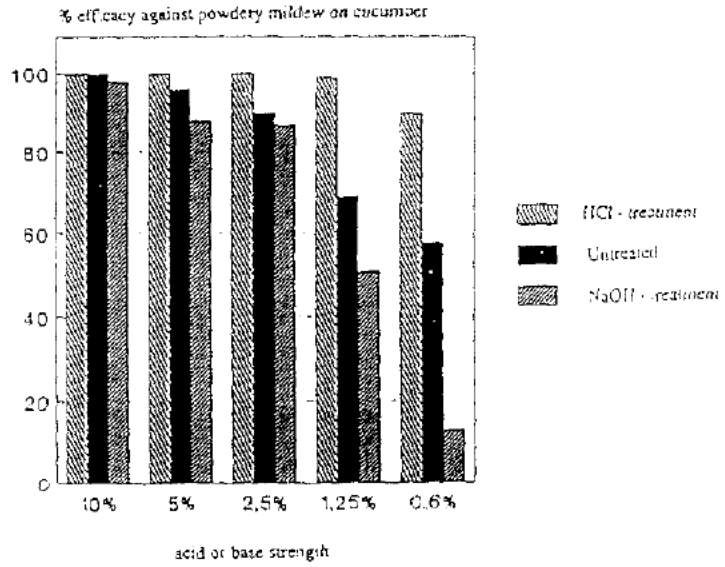


Fig. 7. Hydrolysis and Efficacy (Kowalewski)

This could be interpreted as the possible instability of the active ingredient under alkaline conditions and as the cleavage of a precursor, which releases the active ingredient under acid conditions.

c. Involvement of carbonyl compounds in induction of resistance[10)]

Ten milliliters of the 1% water extract were boiled twice with 0.1 ml phenylhydrazine and 0.1 ml acetic acid for 30 min. The phenylhydrazones that had formed were precipitated by centrifugation. A solution of phenylhydrazine and acetic acid was used as a control in the bioassay. The extract without the carbonyl compounds and the control were tested for their efficacy against powdery mildew on potted cucumber plants.

By boiling the water extract with phenylhydrazine, substances with a free carbonyl group were precipitated (VEIBEL, 1960). The extract, which was free of carbonyl compounds (and phenylhydrazine as a control), did not have any effect on powdery mildew. This indicates that the active substance contains at least one free carbonyl group.

d. Existence of a precursor[10)]

The aqueous extract and squeezed sap of leaves from *R. sachalinensis* were compared in different concentrations on cucumber leaf discs. Polar or somewhat polar substances are often dissolved in the cell plasma. Two ml of the squeezed sap (diluted to 10%) were boiled with 100 µl HCl for 10 min and were tested, also.

Figure 6 shows the efficacy of the water extract in comparison with the squeezed sap from *R. sachalinensis* leaves in different concentrations. This sap was expected to be free of cell wall compounds. The results indicate that the active form of the resistance inducing agent was not dissolved in the sap of the cell.

The inactive sap gained activity after boiling with acid (increase of efficacy from 20% to 89%). Obviously, the active principle was formed out of a precursor. The results indicate that the inactive form in the leaves of the knotweed was activitated during the extraction procedure.

e. Esterification[2] with acetylchloride resulted in the complete loss of biological activity. Subsequent alkaline methanolysis did not replace the initial fungicidal activity of the extract. It remained as biologically inactive as the esterified fraction. Thus the active ingredient must be sensitive to chemical modifications.

f. The phenylhydrazine reaction[2] is a classical proof of ketone and aldehyde groups in molecules. In this case, the phenylhydrazone formation and the removal of the solid components by centrifugation led again to a total inactivation of the supernatant. This is a strong hint that carbonyl- or sugar-type compounds are involved. The HPLC analysis of the product formed by the phenylhydrazine reaction gave a very complex picture which could not be separated.

g. A sugar analysis[2] of the aqueous extract identified two sugars: glucose and fructose. At 7.8% glucose and 7.2% fructose, they represent a relatively high proportion of the dry material. Not surprisingly, a check of commercially available sugars revealed that none of them showed a significant fungicidal activity. These included five sugar-alcohols: ribitol, sorbitol, inositol, mannitol and xylitol; 13 monosaccharides: D-allose, D-xylose, D- and L-arabinose, D-ribose, D-mannoheptulose, D- and L-glucose, D- and L-galaktose, D- and L-fructose and L-mannose; and nine disaccharides: cellobiose, lactose, lactulose, maltose, maltulose, melibiose, isomaltulose, saccharose and trehalose.
Thus the active ingredient cannot be found in the group of simple carbohydrates.

VI. Physicochemical methods

a. According to Lehmann[14], oligosaccharides should be adsorbable to charcoal and elutable with water or ethanol or mixtures of both. In this case, the filtrate through charcoal had no biological activity, nor had the concentrated aqueous and ethanol eluates. Thus the active ingredient is irreversibly bound to charcoal.

b. To get a rough idea of the molecular weight of the active ingredient, an ultrafiltration was made using a membrane selective for 10,000 Dalton. (Figure 8)

ULTRAFILTRATION AND EFFICACY

1 % extract	< 10.000 Da	> 10.000 Da
73 %	24 %	32 %

Fig 8: (Kowalewski)

Neither the filtrate nor the residue reached the fungicidal-inducing activity of the original aqueous extract.

c. A Sephadex G-25 column[2], which absorbs compounds of 100 to 1000 Dalton, was used to check a different range of possible molecular weight. The seven concentrated eluates showed no fungicidal activity. But in this case it was observed that some colored zones did not move on the column at all. Even the addition of 20% ethanol could not elute these zones.

d. Sephadex G-25 and BIO-RAD columns[10]

The extract was fractionated by:

- Sephadex G-25 column with 2M ammonium acetate solution,
- BIO-RAD P2 and BIO-RAD P6 columns with water.

The combined fractions of each column were tested for their efficacy against powdery mildew with the cucumber lead disc test. With different columns, a separation of the extract into fractions of different molecular weight was performed. The eluates from the BIO-RAD P2, the BIO-RAD P6, and the Sephadex G-25 columns showed little or no resistance-inducing activity. As in the reversed-phase column and on the silica gel plates, the active substance was spread or bound to the matrixes.

Other attempts to separate the active ingredient by gel-chromatography[2] also failed since again none of the eluates showed any fungicidal activity. This the active ingredient is obviously tightly bound to the different gel matrices used.

e. Ion exchange[10]

The extract was analyzed by strong ion exchange columns: Amberlite IR-120 (elution with 6% NH_3 solution) and Lewatit (elution with 3N H_2SO_4). The different fractions were tested for their efficacy against powdery mildew on potted cucumber plants. The results of the ion exchange experiments showed that the active ingredient is uncharged.

VII. Conclusions

Isolation or identification of the ingredient or ingredients responsible for the activity of *Reynoutria sachalinensis* has yet to be achieved. In general, studies have excluded the possibility of polypeptides or proteins, terpenoids, phenolic substances, simple or reducing sugars, flavonoids, or steroids. The concensus now is that the compound is probably somewhat polar, amphoteric, and most likely a carbohydrate with a hydrophobic moiety.

In light of the previously cited research, such an effort by any known analytical means appears unlikely to shed further light on the identity of the naturally occurring elicitors found in *Rs* extracts.

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    Note: The "extract" referred to in each case was prepared as follows:

    Air-dried and ground leaves were used for the preparation of a 1% extract: 1 g leaf powder was extracted with 10 ml acetone for 10 min and then water (containing 0.0125% Citowett) was added to a volume of 100 ml. After 1 hour the extract was filtered by suction and used for the bioassay.

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Chemical:	**Reynoutria sachalinensis**
	PC Code: 055809
HED File Code:	**41500 BPPD Tox/Chem**
Memo Date:	**12/21/1999**
File ID:	**DPD257772**
Accession #:	**000-00-9002**

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